Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 4175–4179, May 1994
Biochemistry

Evolution of distinct DNA-binding specificities within the nuclear receptor family of transcription factors

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Communicated by Viktor Mutt, January 10, 1994

ABSTRACT **Nuclear receptors are ligand-activated transcription factors that interact with response elements within regulated genes. Most receptors, typified by the estrogen receptor, have three amino acids within the DNA-binding domain that specify recognition of the sequence TGACCT within the response element. However, in the glucocorticoid group of receptors, these residues have evolved to recognize the sequence TGTTCT. Saturation mutagenesis was used to investigate the role played by two of these residues (Gly-439 and Ser-440 of the human glucocorticoid receptor) in receptor specificity. We conclude that these residues, and their equivalents in the estrogen receptor, play roles unique to the respective amino acids. In the glucocorticoid receptor the side chain hydroxyl group is the important component of Ser-440 that contributes to specificity by inhibiting interaction with estrogen response elements. Several substitution mutants at position 439 interact well with estrogen response elements; therefore, the unique specificity feature of Glu-439, which mimics the estrogen receptor, is its inhibition of interaction with noncognate sites. In contrast to position 440, where most substitutions prevent interaction with DNA, replacements of residue 439 have the potential to contribute to the evolution of DNA-binding specificities within the nuclear receptor family. The liver-enriched HNF-4 and *Drosophila* Tailless transcription factors are known examples of receptors that have diverged at this position.**

Regulation of gene expression involves a large number of transcription factors with unique DNA-recognition properties (1). Many transcription factors belong to families of related proteins, the members of which bind to similar but distinct DNA sequences. Thus, in order to regulate the correct subset of genes, proteins within a family must be able to discriminate between closely related DNA sequences. Several strategies for binding site discrimination have developed during the evolution of DNA-binding specificities within transcription factor families (1). One strategy involves the recognition of alterations in the base-pair sequence of the DNA. In a second strategy, discrimination results from altered spacing or organization of the DNA-binding sites that are then recognized by different homo- and heterodimeric forms of the transcription factors.

These two strategies to accomplish discrimination between closely related DNA sequences are used by the nuclear receptors, which belong to a family of ligand-activated transcription factors including the receptors for steroid hormones, thyroid hormone, retinoids, and vitamin D, and a large number of receptors with unknown ligands or activation mechanisms (2, 3). The receptors bind as dimers to DNA sequences, response elements, which are composed of two 6-bp half-site sequences arranged as palindromic or direct repeats with different spacing between the repeats. The steroid receptors bind as homodimers to palindromic repeats (4, 5), whereas thyroid hormone, retinoic acid, and vitamin D receptors bind with high affinity as heterodimers with the retinoid X receptor to direct repeats (6–11). Most receptors (12), typified by the estrogen receptor (ER), recognize the same primary half-site sequence, TGACCT, with specificity resulting from altered spacing or organization of the half-sites (13–15). However, the glucocorticoid receptor (GR) group, which also includes the progesterone, androgen, and mineralocorticoid receptors, has evolved to recognize an altered half-site sequence, TGTTCT (2).

The DNA-binding function of the nuclear receptors is encoded by the DNA-binding domain (DBD), which is highly conserved within the receptor family. The DBD has two zinc coordination sites, each of which occurs near the N terminus of an α-helix (16–20). The N-terminal α-helix functions as a DNA recognition helix and is placed in the major groove of the DNA when the receptor binds to the response element (19). The amino acids that are involved in discrimination between the TGACCT and TGTTCT half-site variants are encoded by a region called the P-box in the recognition helix (21–23). Mutation of the three P-box residues in the GR or ER to the corresponding residues in the other receptor switches the DNA-binding specificity of the mutant and thus enables it to interact preferentially with the noncognate half-site sequence (21–23). Comparison of P-box sequences from different groups of receptors suggests that the P-box of the GR group has evolved from a progenitor whose P-box resembled that of most present day receptors, including the ER (Fig. 1*A*) (12). Thus, mutations in the P-box during evolution have changed the DNA-binding specificity of proteins that have high affinity for TGACCT half-sites but low affinity for TGTTCT sequences, resulting in proteins that show the opposite specificity.

The mechanism by which the P-box residues contribute to specificity is poorly understood. Only one of the three specificity-determining residues in the GR, Val-443, interacts directly with the DNA (19). This study develops our previous observation that, in addition to positive contacts with cognate sites, some of the discriminatory properties of the P-box residues result from inhibition of binding to noncognate sites (26). Consistent with such a role, P-box residues Gly-439 and Ser-440 of the GR, which are of predominant importance for discrimination (21, 23, 26, 27), have not been shown to interact directly with glucocorticoid response elements (GREs). To investigate the role of these residues in the process whereby the distinct response element specificity of the GR group of receptors evolved, we introduced all possible amino acid substitutions at positions 439 and 440 in the P-box. We tested the specificity of the amino acid substitutions by using a range of response elements and could thus assess the contribution of different amino acid side chains to response element specificity. We conclude that GR residues

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Abbreviations: ER, estrogen receptor; GR, glucocorticoid receptor; DBD, DNA-binding domain; GRE, glucocorticoid response element; ERE, estrogen response element.

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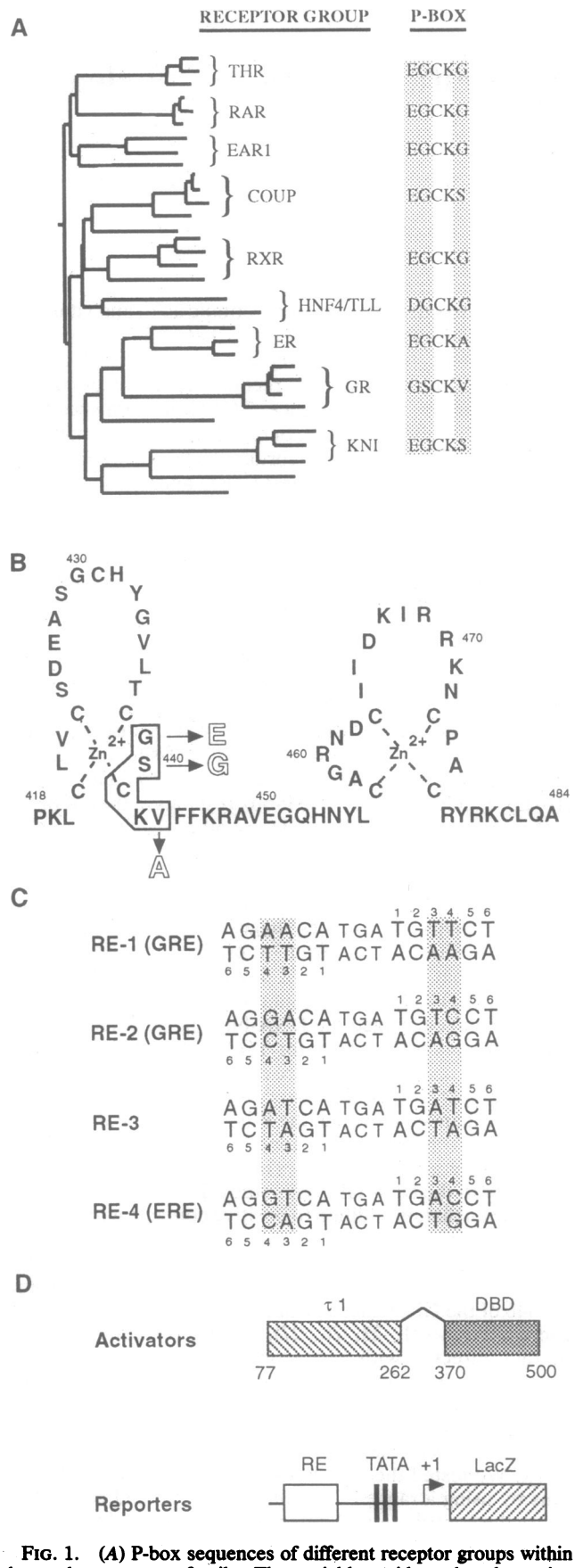

FIG. 1. (*A*) P-box sequences of different receptor groups within the nuclear receptor family. The variable residues that determine specificity are shaded. The phylogenetic tree is adapted from ref. 12. (*B*) Amino acid sequence of the GR EGA DBD, a human GR DBD in which the GR residues Gly-439, Ser-440, and Val-443 in the P-box (boxed) have been replaced with ER residues Glu, Gly, and Ala (shown as open letters E, G, and A), respectively (24). (*C*) Sequence of the response elements used. RE-1 and RE-2 are functional GREs, RE-4 is an ERE, and RE-3 is an intermediate binding site that interacts poorly with both the GR and ER (25). The base pairs constituting each half-site are numbered 1–6 and those that vary between the elements are shaded. (*D*) Activator proteins (τ1-DBD) and reporter genes (*RE-CYC1Δ-LacZ*) used to measure the interaction of mutant DBDs with different response elements in a yeast transactivation assay. τ1 is the major transactivation domain from the human GR. The reporter genes consist of the *LacZ* gene expressed from a basal *CYC1* promoter with the different response elements cloned upstream.

439 and 440 and their equivalents in the ER play roles unique to the respective amino acids and that this is to a large extent associated with their role in inhibiting interaction with noncognate sites. Replacements at position 439 have the potential to contribute to the evolution of DNA-binding specificities within the nuclear receptor family. Interestingly, divergence at this position has been observed in the liver-enriched HNF-4 (28) and the *Drosophila* Tailless (29) transcription factors in addition to the GR group of receptors.

MATERIALS AND METHODS

Codon-Specific Cassette Mutagenesis. Codon-specific cassette mutagenesis was performed by using 133-bp oligonucleotides in which all four nucleotides were incorporated at random in the first two positions of the mutagenized codon and where the third position was restricted to a random choice of guanidine or thymidine. The resulting pool of oligonucleotides thus contains codons at this position encoding all possible amino acid substitutions. The remaining encoded amino acids were from the GR, except for the P-box in which nonmutated residues were from the ER. Full-length oligonucleotides were selectively amplified by PCR using primers that could be cleaved with *Acc* III and *Ecl* XI, thus allowing insertion of the mutagenized cassettes into the corresponding sites in pKV-GRDBD-cas. The mutagenized pool of plasmids was transformed into *Escherichia coli* and individual mutants were identified by sequencing of plasmid minipreps. pKV-GRDBD-cas was constructed by modifying the yeast expression plasmid, pKV-GRDBD (24), that expresses the τ_1-DBD protein as follows. All sites for restriction enzymes *Acc* III and *Ecl* XI were removed from the plasmid. They were then reintroduced in sequences encoding amino acids 429 and 458 of the human GR DBD, respectively, without changing the amino acid sequence of the encoded protein. A linker sequence containing sites for *Xho* I and *Nhe* I was inserted between the *Acc* III and *Ecl* XI sites. The TRP1 yeast selection marker from pJG3 (30) was also introduced.

Transactivation Assay in Yeast Cells. The yeast strain W303-1A (31) was transformed with the pKV-GRDBD-cas plasmids containing mutant DBDs. Transformants were mated with four strains, each consisting of FY 24 (*MATα, ura3-52, trp1-Δ63, leu2-Δ1, GAL*$^+$) containing a reporter plasmid in which the *LacZ* reporter gene is regulated by one of the four response elements (Fig. 1*C*). These reporter plasmids have been described (24). The diploid yeast strains were grown to stationary phase at 30°C in minimal medium containing 3% (vol/vol) glycerol and 1% ethanol as carbon sources and lacking tryptophan and uracil, and then they were diluted with the same medium containing 2% galactose to a density of about A_{600} = 0.3 to induce the expression of the τ_1-DBD protein. The cells were harvested after 5 h, and protein extracts were prepared and assayed for β-galactosidase activity and protein concentration (30). β-Galactosidase activity is expressed as nmol of *o*-nitrophenyl β-*o*-galactoside substrate converted per min per mg of protein.

Immunoblotting. Immunoblots of protein extracts electrophoresed on SDS/15% polyacrylamide gels were performed as described (31) except that a monoclonal antibody raised against the GR DBD was used.

RESULTS

Experimental Approach. As a starting point for mutagenesis of the P-box residues we used a mutant GR DBD protein, GR EGA, that contains the three ER-specific P-box residues (Glu-439, Gly-440, Ala-443) (Fig. 1*B*). This protein shows a preference for binding estrogen response elements (EREs) (24), and thus P-box mutations mimic the process of evolution of the GR group of receptors from ERE to GRE specificity. The substituted residues were mutated to all possible amino acids by codon-specific cassette mutagenesis. The DNA-binding characteristics of the mutant DBDs were determined by using a transactivation assay in the yeast *Saccharomyces cerevisiae*. The mutant DBDs were expressed coupled to the τ_1 transactivation domain of the GR, and their interaction with different binding sites was assayed by using reporter genes consisting of the β-galactosidase gene from *E. coli* coupled to the basal yeast iso-1-cytochrome *c* promoter with different response elements inserted upstream (Fig. 1*D*). The palindromic response elements used were two functional GREs that differ from each other at position 4 in the half-site (RE-1 and RE-2), an ERE (RE-4), and an intermediate binding site that interacts poorly with both GR and ER (RE-3) (Fig. 1*C*) (25). The transactivation assay correlates very well with the *in vitro* DNA-binding specificity of a range of purified mutant GR DBD proteins that we have tested (24, 26). To facilitate comparison of mutants that differ in overall affinity for DNA, we have supplemented the primary data by expressing the assay values for interaction of each mutant with individual response elements relative to the sum of the values for all four response elements. Thus, the relative affinity of the mutant proteins for the different response elements is shown by the size of the shaded rectangles in Figs. 2 and 3.

Response Element Discrimination by Substitution Mutants at Residue 439. The first P-box residue (position 439) is a glutamate in the ER and a glycine in the GR. Substitution of residue 439 with alternative amino acids produced 19 mutant proteins, all of which bound DNA, showing a range of specificities (Fig. 2). Interestingly, glutamate and glycine, found in the ER and GR, respectively, both have unique specificity properties that cannot be mimicked by other amino acids.

The ER-specific Glu-439 mutant shows the highest affinity for the cognate binding site, ERE (RE-4), and a lower affinity for the other response elements. Since several substitution mutants in addition to the Glu-439 mutant interact well with the ERE (RE-4), the unique feature of ER residue Glu-439 is its low affinity for the noncognate binding sites RE-1 (GRE) and RE-3, containing thymine at position 4 (T-4). All other substitution mutants show a higher affinity than the Glu-439 mutant to these two response elements; thus, the inhibitory property is an exclusive function of Glu-439. Since residues of similar size (i.e., Gln) or charge (i.e., Asp) lack this inhibitory action, the important property may be a combination of both the size and polarity of the glutamate side chain.

The GR-specific Gly-439 mutant has a unique specificity profile, suggesting that GR specificity is not merely a default pattern that results in the absence of Glu-439. This is supported by the results for the Ala-439 mutant, which is less discriminatory than Gly-439 even though it too lacks the Glu-439 side chain. Several mutants (i.e., lysine, arginine, threonine, valine, and isoleucine) show similar specificity to the Gly-439 mutant, to the extent that they interact poorly with the ERE (RE-4), but they are not equally GR specific since they interact only with the RE-1 subset of GREs.

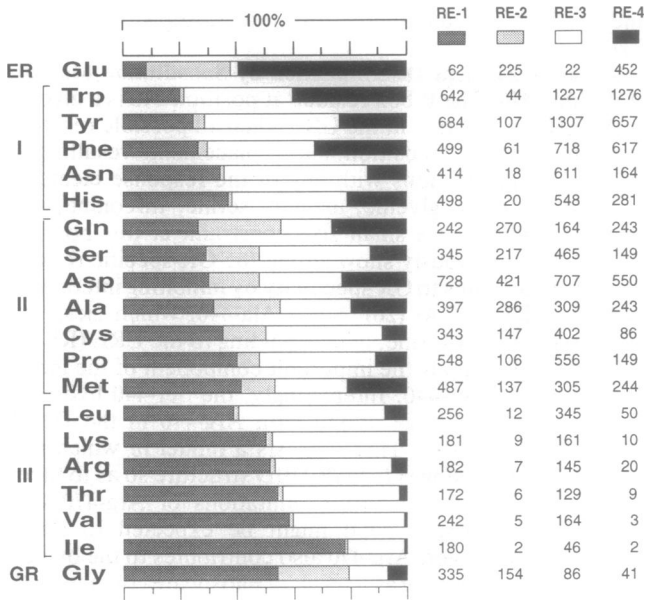

FIG. 2. Response element specificity of substitution mutants at residue 439. The size of the shaded rectangles in the chart represents the relative affinity of substitution mutants for the different response elements (the sum of the activities from all four response elements = 100%). The means of the actual transactivation activities from three experiments are tabulated. SDs were generally <20% of the mean, except in some cases in which the measured values were very low. Classes of substitution mutants showing similar unusual specificities are indicated by Roman numerals.

Substitutions of residue 439 have the potential to create unusual DNA-binding specificities within the nuclear receptor family. In addition to the unique specificities of the glutamate and glycine mutants, the other residues can be divided into three classes with distinct specificity properties. The aromatic residues, tryptophan, tyrosine, and phenylalanine, as well as asparagine and histidine, form a group of mutants that bind well to all response elements except the GRE RE-2 (class I). The glutamine, serine, aspartate, alanine, cysteine, proline, and methionine mutants discriminate poorly between the response elements, which results in a broad binding specificity (class II). The basic residues lysine and arginine as well as threonine, valine, leucine, and isoleucine form a group that interact poorly with RE-2 (GRE)

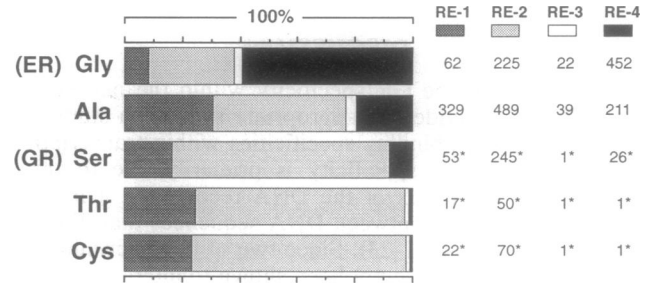

FIG. 3. Response element specificity of substitution mutants at residue 440. Transactivation activity of the proteins was measured and results are presented as described in Fig. 2, except that mutants indicated by asterisks were generally ≈4-fold reduced in activity after the normal 5-h expression period. They were harvested after 7 h so that relative activities for different response elements could be estimated accurately. Thus, the actual measured activities should not be compared directly with other values. Fifteen substitution mutants for which no activity could be measured are not shown.

and RE-4 (ERE), containing cytosine at position 4 (C-4) (class III).

Response Element Discrimination by Substitution Mutants at Residue 440. The P-box residue at position 440 is a glycine in the ER and a serine in the GR. When all possible residues were introduced at position 440, replacements resulting in measurable interactions with any of the response elements were restricted to glycine, alanine, serine, threonine, and cysteine, indicating a small size constraint at this position (Fig. 3). We previously showed that the GR-specific residue Ser-440 contributes to GR specificity by inhibiting interaction with the ERE (RE-4) (26). Since Ala-440, which lacks the hydroxyl group of serine, allows binding to the ERE (RE-4), the hydroxyl group is the important component of the inhibitory action of Ser-440. Interestingly, the Ser-440 hydroxyl group forms a hydrogen bond with Arg-470 in the DNA-bound GR DBD (19). This suggests a model in which this hydrogen bond changes the GR DBD structure so as to favor interaction with GREs. Thus, mutations of Arg-470 that disrupt the hydrogen bond might be expected to affect specificity. However, Arg-470 also contributes to the overall affinity of DNA binding as a result of phosphate contacts with the DNA backbone (19). Thus, mutations of Arg-470 to lysine, threonine, and isoleucine resulted in proteins that did not interact with any of the response elements with sufficient affinity, either in the transactivation assay or in an *in vitro* DNA-binding assay for this prediction to be tested (data not shown). However, the GR specificity shown by the Thr-440 and Cys-440 mutants supports the model since they have side chain hydroxyl and thiol groups, respectively, that have the capacity to replace the Ser-440 hydroxyl group in hydrogen bond formation.

The Thr-440 and Cys-440 mutants have a very low affinity for the ERE (RE-4) (Fig. 3). This apparently better GR specificity of the Thr-440 and Cys-440 mutants relative to the GR-specific residue Ser-440 led us to question why the GR did not evolve with threonine or cysteine replacing Ser-440. To investigate this, we studied mutant GR DBD proteins that were mutated only at residue 440. Mutation of Ser-440 to cysteine or threonine resulted in the same specificity as wild-type GR DBD but showed lower levels of activity (Fig. 4*A*), although all proteins were expressed at similar levels (Fig. 4*B*). This suggests that the presence of Ser-440 in the GR results from the requirements for both specificity and affinity of DNA binding. The ER-specific Gly-440 mutant has a higher affinity for the ERE (RE-4) than the Ala-440 mutant (Fig. 3). This shows that ERE specificity is not just the default pattern observed in the absence of the hydroxyl group of the GR-specific Ser-440. Thus, Gly-440 appears to play a unique role in ER specificity.

DISCUSSION

The evolution of the GR specificity within the nuclear receptor family provides an appropriate system to study the evolution of DNA-binding specificities within transcription factor families. GR specificity is primarily due to three mutations in the P-box of the DNA recognition helix that allow discrimination between DNA sequences that differ at only 2-bp positions (21–23). Since two of the discriminating residues in the GR have not been shown to interact with the DNA, the evolution of specificities appears to involve more than juxtaposition of alternative sets of specifically interacting residues. We have used saturation mutagenesis to study the function of these residues in binding site discrimination. Together with previous data (19, 26) our results contribute to a better understanding of the roles played by the P-box residues in the evolution of the DNA-binding specificity that typifies the GR group of receptors. The results are summarized in Table 1.

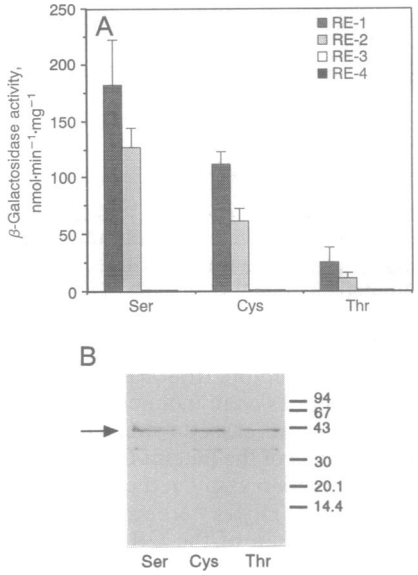

FIG. 4. (*A*) Specificity and activity of Cys-440 and Thr-440 substitution mutants in the normal GR DBD context in relation to wild type (Ser-440). Transactivation activity of the proteins was measured as described in Fig. 2. Mean values + SD from three experiments are shown. (*B*) Level of the expressed wild type and mutant GR DBD derivatives in the transactivation experiments in *A* was analyzed by immunoblot. Migration of molecular size markers is shown (kDa).

The residues at positions 439 and 440 play roles unique to the respective amino acid for the specificity of the GR group of receptors. The GR-specific Ser-440 appears to contribute to specificity by decreasing the binding affinity for EREs (26). Our present results show that the side chain hydroxyl group is the important component of Ser-440 and is consistent with a model in which the hydroxyl group is involved in hydrogen bond formation with Arg-470 (19). This hydrogen bond could reorientate the structure of the DBD to favor specificity for GREs without requiring interaction of Ser-440 itself with DNA. The hydroxyl group of threonine and the thiol group of cysteine at position 440 give the same specificity pattern as the hydroxyl group of Ser-440. However, the Thr-440 and Cys-440 mutants show lower DNA-binding affinity than the wild-type GR DBD, suggesting that GR has evolved with Ser-440 due to requirements for both specificity and affinity. Our results suggest two aspects to the role of the GR-specific Gly-439 in the acquisition of GR specificity. (*i*) Any substitutions of the ER residue glutamate appear to alleviate the

Table 1. Summary of response element discrimination by amino acid substitutions at positions 439 and 440 of the GR

Class	Amino acid	Specificity
	Position 439	
GR	Gly	RE-1 > RE-2 > RE-3 > RE-4
ER	Glu	RE-4 > RE-2 > RE-1 > RE-3
I	Trp, Tyr, Phe, Asn, His	RE-3 ≥ RE-4 ≥ RE-1 ≫ RE-2
II	Gln, Ser, Asp, Ala, Cys, Pro, Met	Poor discrimination between response elements
III	Lys, Arg, Thr, Leu, Val, Ile	RE-1 ≥ RE-3 ≫ RE-4 ≥ RE-2
	Position 440	
GR	Ser (Thr, Cys)	RE-2 > RE-1 ≫ RE-4 ≥ RE-3
ER	Gly	RE-4 > RE-2 ≫ RE-1 > RE-3
I	Ala	RE-2 > RE-1 > RE-4 ≫ RE-3
II	All other amino acids	No DNA interaction detected

Note that the above results were obtained with the GR EGA DBD mutant.

inhibitory role played by glutamate on interaction with GREs (RE-1). (*ii*) Since Gly-439 is the only substitution mutant that recognizes both the GRE sequences (RE-1 and RE-2) with high specificity, it is tempting to speculate that it also plays a positive role specific to glycine. Since glycine is the smallest amino acid it may be that it is the only residue that does not interfere with GR-specific DNA interactions. This is consistent with the observation that Gly-439, while not itself interacting with DNA, lies in very close proximity to amino acid side chains (e.g., Val-443) that do (17, 19).

The specificity of the ER group of receptors is also determined by unique functions of the residues at positions 439 and 440. An important function of the ER-specific Glu-439 is inhibition of binding to the noncognate response elements RE-1 and RE-3 that contain T-4. We previously suggested (26) that Glu-439 might inhibit interaction with these response elements by interfering with a water-mediated hydrogen bond that has been observed between Lys-442 and T-4 of the GRE (19). Our present data suggest that this inhibitory action is an exclusive function of Glu-439, perhaps due to a combination of both its size and polarity. We previously showed (26) that mutants containing Glu-439 showed a high affinity to binding sites containing C-4 and we suggested that this could be due to hydrogen bond formation between Glu-439 and C-4. The ER-specific Glu-439 therefore seems to be involved in recognition of the base pair at position 4 using both positive and negative interactions. The ER-specific Gly-440 plays a unique role in determining ERE specificity that is not only due to the absence of the GR-specific Ser-440 that interferes with ERE binding. Thus, Gly-440 appears to play a positive role for the affinity for EREs. Possibly the presence of side chains from amino acids other than glycine at this position influences the DNA interactions made by other residues in the vicinity, by analogy to the model for the function of Gly-439 for GR specificity.

Of the two positions studied, substitutions at position 439 have the greatest potential to contribute to further diversification of the DNA-binding specificities within the nuclear receptor family, as summarized in Table 1. Notably, many of the mutant proteins (class I and II) interact well with RE-3, which is a poor response element for both the GR and ER (25). Furthermore, a group of receptors (class III) that interact with only RE-1-like, and not RE-2-like, GREs could evolve by substitution of residue 439 with amino acids such as lysine and valine. Interestingly, Asp-439, which leads to a broad binding specificity, is found in the P-box of the liver-enriched factor HNF-4 (28) and in the *Drosophila* Tailless protein (29). Although no systematic study has been made, HNF-4 has been shown to interact with a range of response elements that vary in the third and fourth half-site positions, consistent with the broad specificity of the Asp-439 mutant (28, 32–35).

Our results from saturation mutagenesis show that the residues at positions 439 and 440 of the GR, and their equivalents in the ER, play roles unique to the respective amino acids. Both the GR-specific Ser-440 and the ER-specific Glu-439 contribute to specificity in part by inhibiting interactions with the noncognate binding sites. The important components are the side chain hydroxyl group of Ser-440 and the size and polarity of the side chain of Glu-439. Interestingly, the GR-specific Gly-439 and the ER-specific Gly-440 seem to play positive roles in specificity and do not merely represent default options that occur in the absence of Glu and Ser, respectively. Although other interpretations should not be excluded, the models we propose for the roles of the residues at positions 439 and 440 in acquisition of GR and ER specificity are testable by tertiary structure determination of selected mutant GR DBD proteins.

We thank members of the Steroid Receptor Unit at the Centre for Biotechnology for helpful discussions and comments during this work, Lorenz Poellinger and Sven Pettersson for critical reading of the manuscript, Delta Biotechnology Ltd. for the pKV50 yeast expression plasmid, Fred Winston for the FY 24 yeast strain, and Ann-Charlotte Wikström and Marika Rönnholm for the anti-GR DBD antibody. The work was supported by grants and fellowships from the Swedish Medical Research Council (Grant 2819), the Swedish Natural Science Research Council (Grant K-KV 9756-301), the Swedish Research Council for Engineering Sciences (Grant 92-737), and the Karolinska Institute.

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